Status of the “Mangrove tunicate” Ecteinascidia turbinata (Ascidiacea: Perophoridae) in the Mediterranean Sea

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Running head : *ECTEINASCIDIA TURBINATA* IN THE MEDITERRANEAN

Status of the "Mangrove tunicate" *Ecteinascidia turbinata* (Ascidiacea: Perophoridae) in the Mediterranean Sea

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ABSTRACT

The ascidian Ecteinascidia turbinata *is reported from Maltese waters for the first time. Mature colonies were recorded on artificial substrata at two different sites (and on natural substrata at one of these), 4 km apart, during the summer months. The appearance of this ascidian is expected to be seasonal as the winter temperature in Malta may fall below that required for the maintenance of zooid growth. A second species,* E. moorei, which *was described in 1890 is here confirmed to be the same as* E. turbinata, *meaning that the species has existed in the Mediterranean since at least ca 1880. The possibility that the Mediterranean population is a relic one from warmer periods cannot yet be excluded, so it is best described as being cryptogenic. The species appears to be spreading slowly, perhaps as a result of the rise in surface sea temperature. The Maltese sites offer an opportunity to monitor the species as they are accessible dive sites. This will allow assessment of whether this species remains restricted to these sites, or if it spreads perhaps to impact other species.*

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INTRODUCTION

Ecteinascidia turbinata (Herman, 1882) has attracted much attention from within the biological and medical fields as it has been found to be a source of several metabolites, some of which may act as anti-cancer agents (Rinehart *et al*, 1990). Foremost amongst these is ET-743 which has a unique mode of action, targeting DNA repair (D'Incalci & Galmatini, 2010). ET-743 is now authorised by the European Commission under the name 'Trabectedin' for use to treat ovarian cancer. It is now known that ET-743 is not produced by the tunicate itself but rather is the product of the intracellular bacterium *Endoecteinascidia frumentensis* (Moss *et al*, 2003; Pérez-Matos *et al*, 2007; Elbaz, 2009).

The main world-wide population of *E. turbinata* is centred on the tropical Western Atlantic (Caribbean) region with what are presumed to be satellite populations occurring elsewhere. However, according to Van Name (1945) "Reports of it from Old World localities … are to be mistrusted, as very probably referring to allied species". It is now known that this is the same species which occurs in West Africa at Joal and Dakar in Senegal (Pérès, 1948, 1949), the Cape Verde Islands (Rennie & Wiseman. 1906), the Suez Canal and into the Red Sea (Elbaz, 2009), and there are also populations in the Mediterranean (Pérès, 1958) (Figure 1). This observation has led to the assumption that *E. turbinata* has expanded its area of distribution, probably through human activity, from the Caribbean area but this has not been confirmed. The Mediterranean Sea has long been a major route for maritime traffic and around 680 alien species have successfully colonized it (Galil *et al*, 2014), with 66 alien species colonizing the Maltese Islands alone (Evans *et al*, 2014). It is generally assumed that vessels have facilitated the transport of these species (Zenetos *et al*, 2012), as components of fouling communities on the hulls (Galil *et al*, 2012) and perhaps more recently through ballast water discharge. Either way, harbours have become hotspots for alien species (Hulme, 2009).

Ascidians are particularly good colonisers and many alien ascidians are now found in the Mediterranean, some of which have become invasive (Izquierdo-Muñoz *et al*, 2009). The opening of the Suez Canal in 1869 has allowed immigration from the Red Sea, and another member of the genus, *E. thurstoni* has colonised locations in the eastern Mediterranean presumably through this route (Shenkar & Loya, 2009). This colonisation process has been enhanced by temperature increases in the Mediterranean (Nykjaer, 2009; Raitsos *et al*, 2010). The enlargement of the Canal in 2015 is likely to increase the invasion rate further (Galil *et al*, 2015). It is not completely clear if alien ascidians in the Mediterranean represent a significant threat to indigenous species or if they may result in inconvenience to human activities as has been the case with many other ascidian species across the world (Adams *et al*, 2011).

METHODS AND MATERIALS

Ecteinascidia turbinata was discovered at three sites in Malta. Several colonies of this species were found and photographed on the 10th September 2015 by SCUBA diving at depths of approximately 20 m. The mature colonies (Figure 2) were attached to the wreck of the SS 'Margit' at Kalkara Creek (35.8907° N, 14.5224° E), in the Grand Harbour, Valletta. The wreck lies on a sand and silt bottom and the water was turbid, with visibility estimated at between 3 and 4 meters. The water temperature at 20 meters was measured to be 26°C. This finding prompted a retrospective investigation of photographs recorded at various other sites;

these included images in the authors' libraries or published online by local divers. We also contacted several underwater nature photographers directly to check if they had ever encountered this species but only considered records as 'confirmed' if supported by photographic evidence. Through this exercise, we came across additional images of *E. turbinata* taken on the wreck of the "Tug 2" tugboat off Exiles, Sliema (35.9197° N, 14.4988° E) and from St. George's Bay (35.9264° N, 14.4891° E), with the earliest record being made at the latter site in August 2008 (Table 1). The 'Tug 2' wreck is located some 4 km away from that of the SS 'Margit' and was scuttled as a SCUBA diving attraction in June 2013, so the tunicates appeared on the wreck within 16 months of its sinking. This wreck lies over a sandy bottom at a depth of 21m, with a rocky reef in the vicinity; the area is generally characterised by clear waters with a typical visibility of 15–25m.

Twelve sampled colonies were fixed with 10% formalin in seawater. Some zooids of each colony have been dissected, stained with Masson haemalum and mounted on permanent slides. Taxonomic identification was carried out using original descriptions (Herdman, 1882, 1890, 1891, Van Name, 1945). The specimens have been deposited in the Department of Marine Sciences of the University of Alicante (DCMBA, ET-Mt01-12).

RESULTS

In general the tunicate described here corresponded closely to former descriptions of *E. turbinata* (Herdman, 1882; 1890; Berrill, 1932; Van Name, 1945). However some of the zooids investigated microscopically were similar to the original description of *E. moorei* (Herdman, 1890). This finding explains the disagreement in the published descriptions of *E. moorei* and *E. turbinata* and confirms their synonymy. The most striking features of these colonial tunicates are the bright orange siphons and the translucent tunic of the zooids. The colonies are made of spherical or hemispherical (depending on attachment) bunches of tight erect zooids, attached by a web of anastomosed stolons (Figure 2a and 2b). The zooids from the sample taken from the 'Tug 2' site are 6-15 mm long and have gonads in different stages of development. The tunic is naked and transparent. The siphons are close together, with some of the orange colour remaining after formalin fixation. When contracted, the siphons are still apparent (Figure 2c), (not invaginated as is typical in *E. thurstoni*). The siphonal musculature is marked (not weak as *E. thurstoni*); the transverse muscles do not cross the intersiphonal space (as in *E. thurstoni*). The muscles are not interrupted between the two sides before the eleventh row of stigmata, but extend farther on the right side. In agreement with others (e.g. Van Name, 1921) there are about 40-50 tentacles present in three to four distinct lengths. The prepharyngeal band has a high anterior blade and a posterior ribbon. There is no dorsal indentation. The dorsal tubercle is small and oval (as in *E. thurstoni*), located under the prepharyngeal band (Figure 3a). The dorsal lamina has elongated and flattened languets with or without a membrane (Figure 3a,b).

The branchial sac has 15-16 rows of stigmata on the left side and 16-17 on the right. Flat or cylindrical papillae (Figure 3c) are united by 15-16 longitudinal vessels on each side, but some papillae, such as those closest to the dorsal lamina, lack vessels. The vessel close to the endostyle is made of T-shaped papillae, sometimes arranged two by two. The longitudinal

vessels are very regular, the meshes containing 2-4 stigmata. The gut forms an open loop without a flat secondary curve (unlike *E. thurstoni*) (Figure 2c). The oval stomach shows two wide oblique bands (Figure 2c) and there is a post-stomach. The intestine ascends at first along the dorsal side but does not become parallel to the dorsal lamina (as in *E. thurstoni*). The anus has two lobes at the level of the 6-7th stigmata row. The male gonad is developed with small and numerous vesicles in concentric rings around the ovary (Figure 2c). The sperm duct converges towards the left side of the rectum at the level of 10-11th stigmata row, and ends close to the anus (Figure 3a). Some zooids presented fertilised ovules and one early larva was observed.

DISCUSSION

Classification of *E. turbinata*.

The genus *Ecteinascidia* was formed by Herdman (Herdman, 1882) from specimens obtained during the 'Challenger' expedition. In this initial report, Herdman reported that tunicate samples from Alexandria harbour (Egypt) were the same species, *E. turbinata*, as he had found in the Caribbean. However, he later (Herman, 1890), reported that the tunicate from Alexandria was in fact a related but distinct species, which he named *E. moorei* in honour of Mr T.J. Moore, curator of the Liverpool Free Public Museum, who had passed the sample to him. There is confusion as to the taxonomic status of *E. moorei* as some authors state that *E. moorei* is a junior synonym of *E. turbinata*. Harant (1927a) includes it as a synonym stating that he had studied both and concluded that they were the same species but did not provide the evidence. Later Harant & Vernieres (1933) stated the synonymy but the reference given for this (Van Name, 1930) was incorrect. However, Van Name (1921: 378) makes the following statement about the dorsal lamina in *E. turbinata*: "… a continuous membrane which is extended into a well-developed languet at each transverse vessel. Or it may be described as a series of languets connected by a basal membrane. It lies turned over toward the right side of the body". One of the characteristics that separate *E. turbinata* of *E. moorei* is the development of the dorsal membrane, but in *E. turbinata* the two types of development of the membrane occur.

In Herdman's dichotomic key (1890: 160), the main characters that separate *E. moorei* from *E. turbinata* and *E. thurstoni* are: dorsal languets and connecting ducts are wide and membranous; whereas *E. turbinata* and *E. thurstoni* present dorsal languets and connecting ducts that are narrow and papilliform. Moreover, Kott (1952: 316) adds the following characters to *E. moorei*: zooids clustered into dense hemisphere, vas deferens ends ventral to rectum; whereas in *E. turbinata* and *E. thurstoni*: zooids clustered into irregular mass, vas deferens ends dorsal to rectum. The specimens from Malta can form dense masses of zooids (Figure 2), present dorsal languets and connecting ducts both narrow and wide (Figure 3a-c), and have a spermiduct that ends ventral or lateral to rectum (Figure 3a).

Others treated *E. moorei* as a full species (Garstang, 1891; Sluiter, 1905; Michaelsen, 1918; Harant, 1927b), a few after it had been synonymised (Kott, 1952; Kott, 1964; Por, 1978). To further complicate the matter, Monniot & Monniot (1997) are of the opinion that the *E. moorei* of Sluiter (1905) is in fact *E. thurstoni* while Pérès (1954) considers another species, *E. herdmanni* as young specimens of *E. turbinata*. Our finding that some zooids within the

same colony conform to the descriptions of both *E. turbinata* and *E. moorei,* confirms that *E. moorei* is a junior synonym of *E. turbinata.* This explains why Herdman (1890) apparently changed his mind about the identity of the sample from Alexandria and why there is no presently known population of *E. moorei*. It remains to be seen if *E. moorei*–like zooids also exist in *E. turbinata* colonies in the Caribbean population.

Two different colour varieties have been identified in *E. turbinata* across the range in the Caribbean, Atlantic and the Mediterranean (López-Legentil & Turon, 2007). Colonies occur as "orange" or "white" especially when photographed against a white background and all those in the Spanish Mediterranean so far described have been the white variety. The colonies that we have found were also of the white (translucent) type. Colony colour may be determined by environmental factors as it has been noted that the colour of *E. thurstoni* colonies was dependent on the available light with poor illumination resulting in colourless animals (Por & Dor 1975). The natural situation in which *E. turbinata* is found in its Caribbean range and in the Red sea, is the submerged roots of mangrove trees where ambient conditions are similar to those found in the SS 'Margit' site in Kalkara Creek, where low light levels were caused by a high silt sediment burden with relatively high temperatures; however, in Cuba it is also found in clean water attached to hard coral between 22 and 38 metres depth (Hernández-Zanuy *et al*, 2007), which is closer to the conditions found in St George's Bay and on the 'Tug 2' wreck (Table 1). *E. turbinata* is also found on wrecks in Bermuda (Berrill, 1932).

The status of *E. turbinata* in the Mediterranean

It is now evident that *E. turbinata* has existed in the Mediterranean since at least the late 1800s but was listed as a non-indigenous species (as *E. moorei*) in the Mediterranean (Streftaris *et al*, 2005) and as alien in the WoRMS database (Sanamyan, 2015). However, it was not included in the lists of recent non-indigenous ascidians by Izquierdo-Munoz *et al*. (2009) and Zenetos *et al*. (2010) on the basis that there are old records of this species and that it is circumtropical. On the other hand, some have described *E. turbinata* as a "probably native species" (Chebbi *et al*, 2010). The EASIN database gives this species as 'cryptogenic' and we feel that this is the safest description. It is also possible that *E. turbinata* survives as relic populations such as those we have found in Malta, from warmer periods in the distant past.

Distribution of *E. turbinata* in the Mediterranean and neighbouring areas

E. turbinata has been described as being rare in the Mediterranean (Pérès, 1949) but it is known (Figure 1) from many localities as follows. The Western Mediterranean: Balearic Islands (Rodriguez, 1922; Pérès, 1957; Ramos *et al* 1991; Carballo *et al* 1997), Alicante (Cornet & Ramos, 1980; Ramos, 1988; Luján, 1995), and Marsala in Sicily (Pérès, 1954) and Bizerte in Tunisia (Pérès, 1956). Central Mediterranean (Ionian Sea) in southern and eastern Tunisia, in the Gulf of Gabès (Pérès, 1954, 1956; Zaouali, 1976; Ben Mustapha *et al*, 2002; Meliane, 2002; Chebbi *et al* 2010), and Kuriat Islands (Ramos & Ben Mustapha, 2010). Aegean Sea (Monniot, 1983; Koutsogiannopoulos *et al*, 2012). With respect to the Levantine basin (Eastern Mediterranean), apart from the old records from Alexandria (Herdman, 1882, 1891; Harant, 1939), no further citations have been made, despite a number of studies carried out in the area (Sedra & Khalil, 1972; Abdel-Messeih, 1982; Ghobashy & Abdel-Messeih, 1991; Ramadan *et al*, 2006; Shenkar & Loya, 2009). Regarding the NW Mediterranean,

Harant (1927a) reported *E. turbinata* from Cap Creus (NE Spain). However, no later studies in this Mediterranean sector have observed this species (Cornet & Ramos, 1980; Ramos, 1984; Lafargue *et al,* 1986; Turón, 1987). *E. turbinata* has also been reported in areas neighbouring the Mediterranean Sea (Fig. 2): the Bay of Cadiz (Ramos, 1988; Ramos *et al*, 1992; Naranjo, 1995), quite near to the Gibraltar Strait; and the Suez Canal and the Red Sea (Harant, 1927b; Elbaz, 2009).

Prospects for the Mediterranean *E. turbinata* populations

Despite the fact that *E. turbinata* larvae are relatively large (4.5 mm), active swimmers that are protected to some extent from fish predation through toxins and aposematic colouration (Young & Bingham, 1987), long range dispersal of this tunicate is reported to be primarily due to rafting of adult colonies (Bingham &Young, 1991), with larval dispersal limited to a few hundred metres (Carballo, 2000). The nearest previously known location of *E. turbinata* to Malta is in the Gulf of Gabès, Tunisia (Figure 1). However it is not likely to have spread to Malta from this location due to the limited autonomous larval dispersion and the hydrographic isolation of Gulf waters. It is possible that this tunicate may have been transported to its present Maltese site by shipping, as two other ascidians are proposed to have done (Evans *et al*, 2015), either in ballast water or as adult colonies with fouling assemblages (Izquierdo Muñoz *et al*. 2009).

Whereas *E. turbinata* colonies grow all year round in the El Bibane lagoon, Tunisia (Chebbi, 2010; Chebbi *et al*, 2010), populations in Alicante and the island of Formentera are seasonal (Luján, 1995; Carballo, 2000) and survive winter months as quiescent bodies derived from the stolons. The crucial temperature below which zooids regress appears to be around 17–18^{o}C (Carballo *et al*, 1997). It remains to be determined if the recently discovered colonies are seasonal in Malta, where fertilised ova and larvae have been observed in late October; in Alicante this happens a month before, in late September (Luján, 1995). However, the data for the SS 'Margit' site (Table 2) indicate that the temperature drops below the threshold for the winter months making it very likely that *E. turbinata* is seasonal. It is of course possible that this particular population has adapted to growth in colder waters (but we note that this has not occurred in the Spanish populations). The colonies present at the Peloponnese site in Greece were large and numerous in July 2011, but very small and sparse in July 2012 (Koutsogiannopoulos *et al*, 2012), indicating that conditions (probably temperature) result in these populations too being seasonal and perhaps often marginal.

CONCLUSIONS

We have recorded several live colonies of *E. turbinata* on two Maltese shipwrecks and in one case in a neighbouring inlet. This is the first report of this ascidian in Maltese waters. All our records so far have been made in the summer months (mid-July to late October) but we suspect that this species will reproduce seasonally around Malta due to the winter water temperatures falling below the 17-18^{o}C threshold below which zooids regress to resting stolons. We expect that conditions will prove suitable for *E. turbinata* across Malta and that

the 'Tug 2' site will be particularly valuable for monitoring its establishment success over time and studying how the presence of this tunicate influences existing species assemblages. Since around 2009, *E. turbinata* seems to be expanding into new areas, and it has now colonised *Posidonia oceanica* meadows and the infralittoral rock of Mar Menor, a coastal lagoon in SE Spain outside the previously recorded areas (A.A.R.E. pers. obs.), the Aegean Sea (Koutsogiannopoulos *et al*, 2012) and Malta (present work). The question as to the origins of the present day populations of Mediterranean *E. turbinata* may be resolved by phylogeographic analysis (López-Legentil & Turon 2005; Elbaz, 2009) comparing variations in suitable genes from across its present range. The available information indicates that the status of *E. turbinata* in Malta and the Mediterranean is best described as being cryptogenic, and yet the possibility of it being a relic population cannot be ruled out.

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553 **Table 1.** A summary of the sites at which *Ecteinascidia turbinata* was recorded in Malta.

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Date	Site	Conditions
17th August 2008	Old boat mooring line at St. George's Bay. Depth 6 m.	Good visibility (15-20m)
12th July 2014	'Tug 2' wreck. Depth 15–20m.	Good visibility (15-20m)
10th October 2014	Exiles reef St Georges Bay. Depth 10–15m.	Good visibility (15-20m)
18th October 2014	'Tug 2' wreck. Depth 15–20m.	Good visibility (ca. 20m)
18th July 2015	'Tug 2' wreck. Depth 15–20m.	Good visibility (15-20m)
10th September 2015	Wreck of SS 'Margit', Kalkara creek, Valletta Harbour. Depth 20 m.	Low light, high suspended particle/silt. Temp 26°C
28th October 2015*	'Tug 2' wreck. Also on a mooring block c200m from wreck. Depth 15–20m.	Good visibility (15-20m) Temp 22°C

555 * samples taken

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563 **Table 2.** Temperature and salinity measurements from outer Kalkara Creek, site of the wreck
564 of the SS 'Margit' measured in 2010 (Gianella Pisani, unpublished).

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Date	Temperature (°C)		Salinity (ppt)	
	Surface	at 5 meters	Surface	at 5 meters
January	16.4	16.1	37.4	37.6
April	18.5	18.4	37.3	37.2
July	28.0	27.9	38.1	38.3
December	20.4	20.2	41.5	41.5

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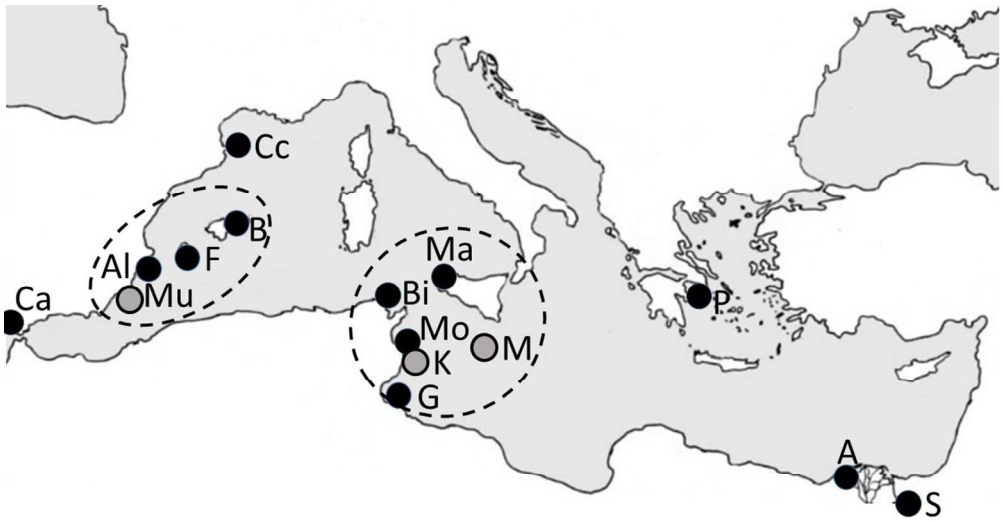
Figure 1 A map of the Mediterranean Sea and the immediate areas, showing the positions of known *Ecteinascidia turbinata* populations (Black circle - previous reports, Grey circles - this study). Two main areas are highlighted (dashed ovals). **A**, Alexandria, Egypt (Herdman 1880); **Al**, Alicante (Cornet & Ramos, 1980; Carballo *et al*, 1997); **B**, Balearics, Spain including Majorca (Ramos *et al*, 1991; Carballo *et al* 1997); **Bi**, Bizerte; **Ca,** Cadiz (Ramos, 1988; López-Legentil & Turon, 2007); **Cc**, Cape Creus (Harant & Vernières, 1933) ; **F**, Formentera Island (Ramos *et al*, 1991; Carballo *et al*, 1997); **G**, Gulf of Gabès (Pérès & Picard, 1956; Ben Mustapha *et al*, 2002); **Mo**, Monastir (Ben Mustapha *et al*, 2002); **K**, Kuriat Island, Tunisia (this study); **M**, Malta (this study); **Ma**, Marsala (Pérès, 1954); **Mu**, Murcia, Spain (this study); **P**, Argolikos Gulf, Peloponnese, Greece (Monniot, 1983; Koutsogiannopoulos *et al*, 2012); and **S**, the Suez Canal (Harant, 1927; Elbaz, 2009).

Figure 2. a,b) Two mature colonies of the ascidian *Ecteinascidia turbinata* photographed on the wreck of the SS 'Margit' in Kalkara Creek, Grand Harbour, Malta on 10 September 2015 (left), and a sample taken from "tug 2" on 28th October 2015 (right). c) A single mature zooid with testes and ovary. The external appearance of the colonies match the differential descriptions for the genus (Goodbody & Cole, 2006). The main distinguishing features include the close position of the orange coloured exhalant and inhalant siphons at the top of the zooid, the lack of red pigment spots between the lobes of the siphon edge and the relative straightness of the intestine (Berrill, 1932). Each individual zooid in both photographs is between 2 and 3 cm in length.

Figure 3. Branchial sac of *Ecteinascidia turbinata* from Malta: a) anterior-dorsal part of the zooid showing perypherical band (bp), dorsal tubercle (dt), neural gland (ng), dorsal languets (dl), anus (a) and sperm duct (sp.); b) dorsal continuous membrane (dm) with languets (dl); c) papillae (p) cylindrical and triangular. Bars: 1mm (a); 0.5mm (b,c).



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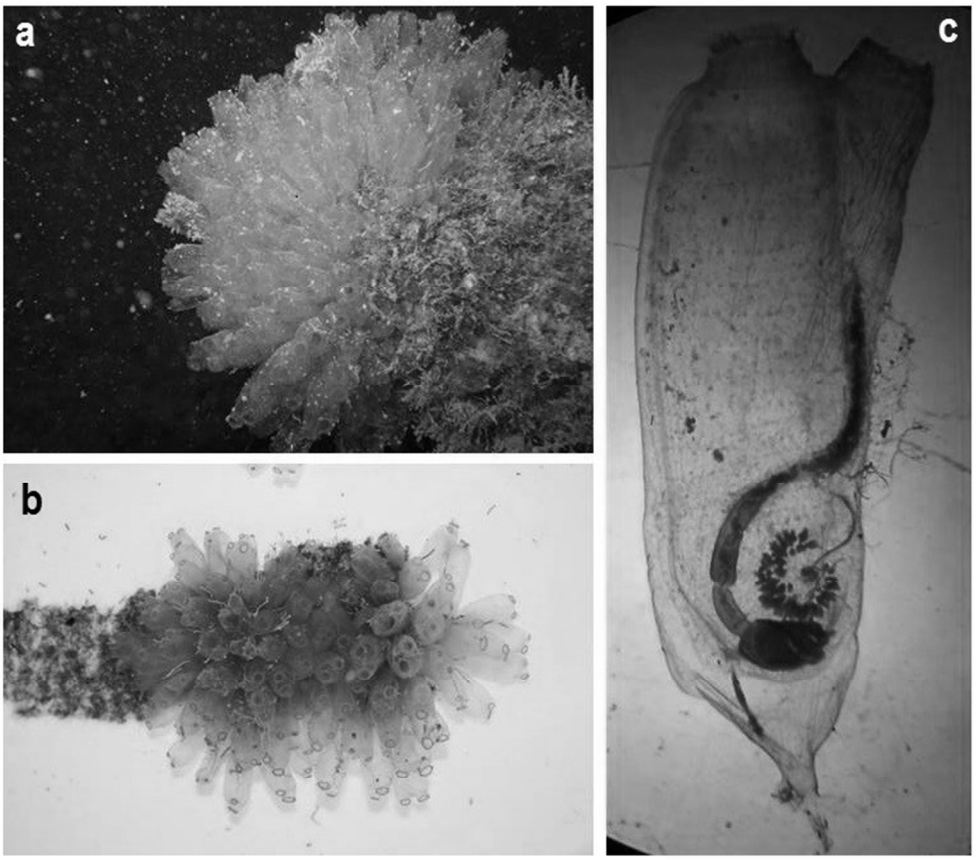

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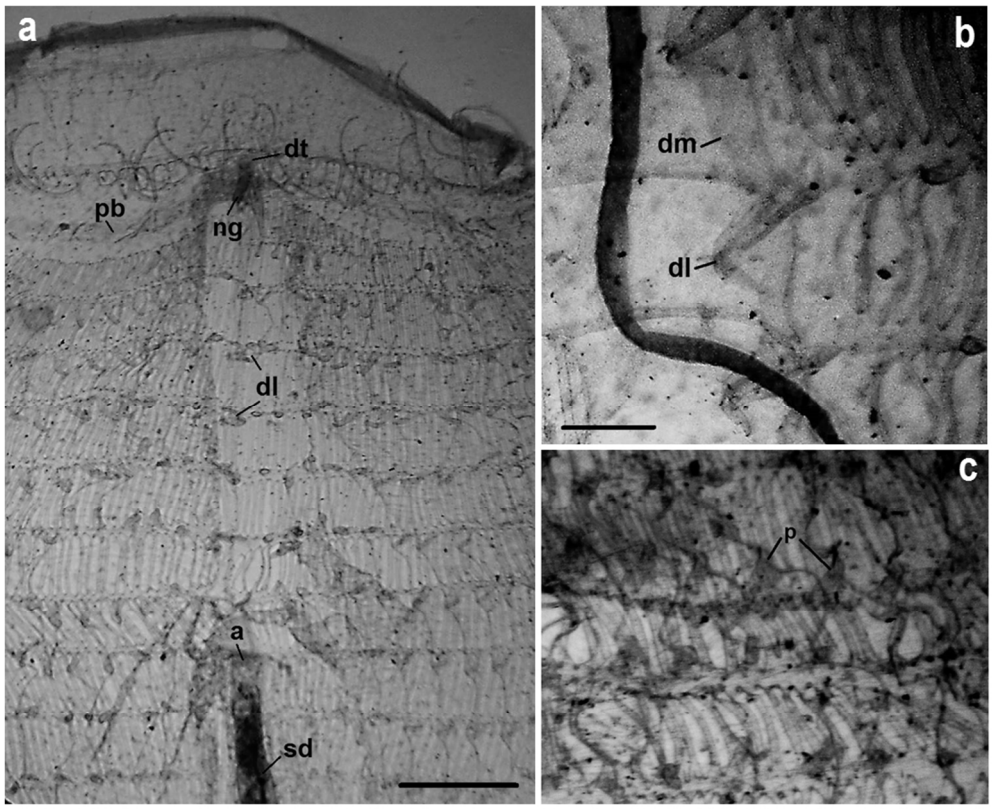

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